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Dichloran-Glycerol Medium for Enumeration of Xerophilic Fungi from Low-Moisture Foods

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A low water activity (a_w) medium (0.95 a_w) containing 18% (wt/wt) glycerol and 2 μg of dichloran per ml was developed for enumerating the fungal flora of dried and semidried foods. The medium, designated DG18, was shown to be significantly better than Christensen malt salt agar when both media were tested with foodstuffs and with pure culture inocula. The need for a medium of reduced a_w for enumerating xerophilic fungi from low-moisture foods was demonstrated by comparing fungal counts obtained on both high-a_w and low-a_w media.

Media used in the enumeration of fungi in foods and other substrates have traditionally been of high water activity (a_w), in the range of 0.999 to 0.997. Although such media are satisfactory for enumerating and isolating yeasts and molds from fresh foods such as fruit, vegetables, dairy products, and meat, in our experience they are inadequate for sampling the fungal flora of dried and semidried foods such as dried fruits, condiments and spices, confectionery, dried meat and fish products, and stored cereals and nuts.

Media of reduced a_w have been in use for many years for the isolation and enumeration of osmophilic yeasts. Ingram (4) noted that as early as 1929 Lockhead and Heron used media containing large proportions of honey for isolating yeasts from that product. Later workers have used media based on glucose and sucrose for the cultivation of osmophilic yeasts (4, 5, 7, 12).

Reduced a_w media for enumerating molds in foodstuffs have traditionally been based on NaCl (1-3, 8, 16, 17), although some workers have preferred to use sucrose-based media (9, 10, 15). The most widely used salt-based medium, Christensen malt salt agar (MSA) (1), is unsatisfactory as an enumeration medium for several reasons. Sodium chloride reacts with agar to produce a soft, granular medium which is difficult to inoculate by the spread plate technique. Depending on the source of the malt extract, the finished medium can be translucent, rather opaque, or contain a brownish precipitate which can obscure small colonies. At the a_w of MSA (0.95), colonies of the *Aspergillus glaucus* group (imperfect states of the genus *Eurotium* Link ex Fr.) spread rapidly, overgrowing some of the more slowly developing species. They also sporulate abundantly, often causing problems with secondary colonies if plates have to be incubated for more than 5 days. Sugar-based media are clear and free of precipitates, but have the disadvantage of high viscosity and gelling temperature and are prone to crystallization.

Pitt and Hocking (11) showed that glycerol is a suitable solute for the cultivation of a range of xerophilic fungi. It is less inhibitory than NaCl to some species, produces transparent media, and is more readily handled than sugars are at high concentrations. However, even media formulated with glycerol do not solve the problem of controlling the spreading growth of the *A. glaucus* group at lowered a_w.

Dichloran (2,6-dichloro-4-nitroaniline), alone and in combination with rose bengal, has been shown to inhibit spreading of mucoraceous fungi and to limit colony diameters of other genera in a fungal enumeration medium for foods (6).

This study describes a new medium containing 18% (wt/wt) glycerol and 2 μg of dichloran per ml (0.955 a_w), developed for the enumeration of xerophilic fungi from dried foodstuffs such as cereals and cereal products, nuts, condiments and spices, dried fruits, and dried meat and fish products.

MATERIALS AND METHODS

Culture media. The new culture medium described and assessed here is dichloran glycerol agar (DG18) with the following formulation: glucose, 10 g; bacteriological peptone (Oxoid), 5 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 15 g; and distilled water to 1 liter. After steaming for 30 min, 220 g of glycerol (analytical reagent grade) and 1 ml of a 0.2% (in ethanol) solution of dichloran were added, giving a final concentration of 18% (wt/wt) glycerol and 2 μg of dichloran per ml. The medium was then sterilized by autoclaving for 15 min at 121°C. Filter-sterilized 0.1% aqueous chlortetracycline (Aureomycin, Lederle) was

added before pouring to give a final concentration of 5 μg/ml. Dichloran was obtained from Tokyo Kasei Co. Ltd., Tokyo, Japan. The final pH of DG18 was 5.6, and the a_w was 0.955.

Two other media were used for comparison in assessing the performance of DG18 as an enumeration medium. These were MSA (1) and dichloran-rose bengal-chlortetracycline agar (DRBC) (6). MSA contained 7.5% (wt/wt) NaCl, 2% malt extract, and 2% agar (pH 4.0 to 5.0, a_w 0.95). DRBC was formulated as follows: glucose, 10 g; bacteriological peptone (Oxoid), 5 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 15 g; distilled water, 1 liter; final pH 5.6; a_w 0.997. Before autoclaving, 0.5 ml of a 5% (wt/vol) aqueous solution of rose bengal and 1.0 ml of a 0.2% (in ethanol) solution of dichloran were added, giving final concentrations of 25 and 2 μg/ml, respectively. Chlortetracycline (0.1% aqueous) was added before pouring at a final concentration of 10 μg/ml.

Inocula. The media were assessed initially by inoculating them with foodstuffs. Samples of dried fish were purchased from local Asian foodstores; mold-affected dried chillies were obtained from the Commonwealth Scientific and Industrial Research Organisation, Division of Entomology, and mold-affected spices were obtained from various household sources. Dried fish and dried chillies were homogenized for 30 s in 0.1% peptone water in a Colworth Stomacher (14), and spices were shaken periodically for 0.5 h with 0.1% peptone water (13). Samples were serially diluted 1:10 in 0.1% peptone water and then surface plated, 0.1 ml/plate, in duplicate.

Pure culture inocula. The following isolates were studied in recovery experiments: *Eurotium rubrum* FRR 1968 (FRR denotes the culture collection of the Commonwealth Scientific and Industrial Research Organisation, Division of Food Research, North Ryde, New South Wales, Australia), *E. umbrosum* FRR 1967, *E. echinulatum* FRR 1661, *E. amstelodami* FRR 475, *E. manginii* FRR 474, *E. chevalieri* FRR 547, *E. repens* FRR 321, *Aspergillus restrictus* FRR 2176, *A. penicilloides* FRR 2178, *Scopulariopsis halophilica* FRR 2184, *Scopulariopsis* sp. FRR 2186, *Wallemia sebi* FRR 1471, *Xeromyces bisporus* FRR 1522, and *Chrysosporium fastidium* FRR 77.

To determine the average colony diameters of a range of xerophilic fungi on DG18, MSA, and DRBC and the relative rates of recovery on the three media, spore suspensions of the cultures were surface plated, 0.1 ml/plate, in triplicate at concentrations ranging from 20 to 500 spores per plate. Spore suspensions of the cultures were prepared in 0.05% Tween 80. The number of spores in each suspension was assessed by total count in a hemacytometer, and appropriate dilutions were made in 0.1% peptone water. The diameters of six colonies of each isolate on each medium were measured. The influence of *Eurotium* colonies on the recovery of some slower-growing xerophiles was also assessed.

All plates were incubated at 25°C. DRBC plates were counted or measured at 4 days. DG18 and MSA plates were examined at 4 days, but counted or measured at 5 and 6 days. Fungi were classified visually into the groups listed in Tables 1 and 2.

RESULTS

Comparison of fungal counts from foods. In comparing the relative performances of DG18 and MSA as enumeration media, a total of 54 counts were carried out on 33 different samples of dried fruits, spices (whole and ground), dried fish and meat products, and cereals. Table 1 shows the results of 15 counts on 10 different commodities. In most cases, DG18 produced slightly higher total counts of fungi than MSA. An analysis of variance of the results from 20 representative counts showed that the higher counts on DG18 were statistically significant ($P \leq 0.05$).

The fungi most commonly isolated from the samples examined were *W. sebi* and species from the *A. restrictus* and *A. glaucus* groups. DG18 generally produced higher counts of *W. sebi* and *A. restrictus* species than MSA, but counts of species from the *A. glaucus* group were sometimes slightly higher on MSA than DG18. Several unidentified xerophilic fungi were isolated during these experiments, and they grew better on DG18 than MSA.

To check that a special low-a_w medium was really necessary for enumerating the fungal flora of dried commodities, counts were also carried out on a high-a_w medium, DRBC (6). In comparisons between DRBC and DG18, 73 counts were carried out on 60 different samples of dried foodstuffs including whole and ground spices, dried meat and fish products, dried fruit, oil seeds, cereals, and cereal products. Some representative data from 14 counts of nine commodities are shown in Table 2. The counts obtained on DG18 were significantly higher than those on DRBC ($P \leq 0.01$), the differences ranging from less than 10^1 to greater than 10^6 in extreme cases. Table 2 shows that the species responsible for these differences in counts were *W. sebi* and species from the *A. restrictus* group. Counts of *A. glaucus* species were often almost as high on DRBC as on DG18.

Results with pure culture inocula. Table 3 shows the relative recovery rates from spores inoculated onto the three test media. There is no significant difference between the percentage of recovery on DG18 and MSA ($P \leq 0.05$), but the difference between DG18 and DRBC is significant. Rates of recovery were generally quite high, except for some of the *Eurotium* species and for *S. halophilica*. However, where rates of recovery were low, DG18 usually gave better results than MSA. For *E. umbrosum* and *E. manginii*, recovery rates on the three media were not significantly different.

The control of colony diameter by DG18 is

TABLE 1. *Comparison of viable counts obtained on DG18 and MSA from various dried foods*

Food	Medium	Viable counts					
		A. glaucus group	*A. restrictus* group	*W. sebi*	Other xerophilic	Other non-xerophilic	Total
Fish flour	DG18		6.0×10^3	2.5×10^2			6.2×10^3
	MSA		5.0×10^3	0			5.0×10^3
Dried fish 1	DG18		5.4×10^7				5.4×10^7
	MSA		5.1×10^7				5.1×10^7
Dried fish 2	DG18				1.8×10^5		1.8×10^5
	MSA				1.7×10^5		1.7×10^5
Semolina[a]	DG18	2.5×10^5	1.2×10^7	2.1×10^4			1.2×10^7
	MSA	2.3×10^5	9.6×10^6	6.5×10^3			9.8×10^6
Chillies	DG18	3.0×10^4	5.3×10^6	2.1×10^4			5.6×10^6
	MSA	1.5×10^4	6.5×10^6	6.5×10^3			6.7×10^6
Black pepper	DG18	3.5×10^4	1.8×10^5			4.7×10^4	2.6×10^5
	MSA	3.1×10^4	1.2×10^5			3.7×10^4	1.9×10^5
Cayenne[a]	DG18	7.0×10^3	5.9×10^6	5.8×10^5			6.5×10^6
	MSA	3.0×10^3	4.9×10^6	1.2×10^5			5.0×10^6
Chilli powder	DG18			1.3×10^9			1.3×10^9
	MSA			1.7×10^9			1.7×10^9
Paprika[b]	DG18	2.1×10^6	1.9×10^6				4.0×10^6
	MSA	2.2×10^6	1.3×10^6				3.5×10^6
Mixed spice	DG18	0	5.6×10^4	5.0×10^2	6.6×10^4		1.2×10^5
	MSA	5.0×10^2	3.1×10^4	1.5×10^3	0		3.3×10^4

[a] Average of three counts.
[b] Average of two counts.

also illustrated in Table 3. Colony diameters of *Eurotium* species on DG18 were smaller than on MSA for all isolates examined, colonies of some species on DG18 being less than half the size of colonies on MSA. Colony diameters of the other four more slowly growing species examined are similar on both DG18 and MSA. The data for colony diameters on DRBC show that a number of species are unable to grow on DRBC at all because of its high a_w, although *A. restrictus* and some *Eurotium* species grow quite vigorously.

This ability of DG18 to restrict colony diameters of *Eurotium* species is important in mixed cultures such as those obtained from foodstuffs, where overgrowth of slower-growing species by eurotia can lead to lowered recoveries. This effect was demonstrated when *E. amstelodami* spores were mixed with spores of *A. penicilloides*, *S. halophilica*, a xerophilic *Scopulariopsis* species, or *W. sebi* (Table 4). Even though the numbers of *Eurotium* colonies were low, counts of the slowly growing xerophiles were significantly less on MSA than on DG18.

DISCUSSION

A comparison of DG18 with MSA and DRBC showed that DG18 was the most suitable medium for enumerating xerophilic fungi from a range of dried foods. Because dichloran controls the colony development of species in the *A. glaucus* group (*Eurotium* species) with less inhibition of the development of more slowly growing species, counts of fungi from foodstuffs on DG18 are generally higher than on MSA. These higher recoveries on DG18 are not reflected in experiments with pure culture inocula because there is no competition between *Eurotium* species and other fungi. When spores of slowly growing species such as *A. penicilloides*, *W. sebi*, or *S. halophilica* were mixed with spores of a *Eurotium* species, then plated on DG18 and MSA, total counts were higher on DG18 than MSA, the difference being due to a higher count of the slowly growing species.

The need for a low-a_w medium for counting the fungi present in low-moisture foods is obvious. In this study, two of the fungi commonly

TABLE 2. *Comparison of viable counts obtained on DG18 and DRBC from various dried foods*

Food	Medium	Viable counts					
		A. glaucus group	*A. restrictus* group	*W. sebi*	Other xerophilic	Other nonxerophilic	Total
Dried fish 1[a]	DG18		3.9×10^7			0	3.9×10^7
	DRBC		1.1×10^4			1.0×10^3	1.2×10^4
Dried fish 2[a]	DG18				3.3×10^5		3.3×10^5
	DRBC				2.6×10^5		2.6×10^5
Christmas pudding (moldy)	DG18	4.8×10^7					4.8×10^7
	DRBC	4.2×10^7					4.2×10^7
Fish flour	DG18		6.0×10^3	2.5×10^2			6.3×10^3
	DRBC		0	0			0
Semolina[b]	DG18	2.5×10^5	1.2×10^7	1.3×10^4			1.2×10^7
	DRBC	1.6×10^5	2.2×10^4	3.3×10^3			1.8×10^5
Chillies	DG18	3.0×10^4	5.3×10^6	3.0×10^5			5.6×10^5
	DRBC	2.0×10^4	2.0×10^4	0			4.0×10^4
Black pepper	DG18	3.5×10^4	1.8×10^5			4.7×10^4	2.6×10^5
	DRBC	2.0×10^4	0			4.4×10^4	6.2×10^4
Cayenne[a]	DG18		5.3×10^6	4.5×10^5			5.8×10^6
	DRBC		0	0			0
Paprika	DG18	3.0×10^6	3.3×10^6				6.3×10^6
	DRBC	7.0×10^5	4.1×10^6				4.8×10^6

[a] Average of two counts.
[b] Average of three counts.

TABLE 3. *Recovery and colony diameters of pure culture inocula on three media*[a]

Species	% Recovery, 5 days[a]			Colony diam (mm), 5 days[b]		
	DG18	MSA	DRBC	DG18	MSA	DRBC
E. rubrum	10.0	9.3	0	16.0	32.3	<1
E. umbrosum	22.0	18.9	20.4	15.2	20.2	1.5
E. echinulatum	58.3	59.8	51.1	15.8	25.3	1.0
E. amstelodami	88.9	67.5	55.5	7.5	24.3	1.0
E. manginii	12.4	12.9	14.4	12.7	25.2	2.5
E. chevalieri	28.2	30.4	20.9	10.7	16.8	3.5
E. repens	63.0	57.3	21.6	14.2	21.2	<1
A. restrictus	83.3	81.0	71.3	6.5	6.6	2.8
A. penicilloides	99.0	97.2	0	2.4	3.4	0
W. sebi	73.3	76.0	0	2.1	1.3	0
S. halophilica	30.8	7.7	0	2.1	2.4	0

[a] Calculated from mean of six counts.
[b] Mean diameter of six colonies.

isolated from dried foods in high numbers (10^6 to 10^9), *A. penicilloides* (a member of the *A. restrictus* group) and *W. sebi*, grow very poorly or not at all on high-a_w media such as DRBC. Counts on high-a_w media of foodstuffs heavily contaminated with either or both of these fungi can be erroneously low. Such foodstuffs could be passed for human consumption even though heavily contaminated with mold.

DG18 agar, although restricting colony diameters of *Eurotium* species, allows near normal growth of most other common xerophilic fungi. Some fastidious xerophiles, such as *X. bisporus* and *C. fastidium*, will not grow on DG18, but neither will they grow on MSA. The physical characteristics of DG18 make it a more suitable enumeration medium than MSA. It is clear and transparent and sets to a firm agar gel. These

TABLE 4. *Effect of E. amstelodami colonies on colony numbers of four slowly growing xerophiles on DG18 and MSA*

E. amstelodami + xerophile	Avg no. of colonies, 5 days[a]	
	DG18	MSA
E. amstelodami + *A. penicilloides*	7 + 76	12 + 52
E. amstelodami + *S. halophilica*	7 + 49	9 + 19
E. amstelodami + *Scopulariopsis* sp.	8 + 105	9 + 48
E. amstelodami + *W. sebi*	7 + 53	8 + 43

[a] Calculated from mean of eight counts.

characteristics, together with improved recovery of molds from dried foods, make DG18 the most suitable of the media tested for enumerating the fungal flora of a wide range of low-moisture and intermediate-moisture foods.

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